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Master Thesis

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**Abstract**

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## Chapter 1

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# Introduction

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### 1.1 The Challenge of Environmental Pollution

Over the past few decades, the upsurge in environmental pollution by chemical compounds has been driven by industrial processes, agricultural methods, consumerism and various other contributing factors. Although these chemicals are integral for many products and have the potential to improve the comfort of modern society, they can also pose risks and adversely affect both human health and the environment, either acutely or chronically. Toxic substances threaten wildlife but also make air, soil, drinking water and food supply less safe.

Nations worldwide maintain comprehensive chemical regulations<sup>1</sup>, however, it is anticipated that global chemicals production will double by 2030 [1]. Moreover, the widespread utilization of chemicals, including their inclusion in consumer goods, is expected to expand further. Even though there are over 275 million known chemical compounds registered by the *Chemical Abstracts Service* [2], merely a tiny fraction of them undergo close monitoring via target analytical approaches and even less is known about their toxicity profiles and negative health effects on organisms. Table 1.1 provides an overview of omnipresent water pollutants.

In light of the rapidly evolving chemical landscape, there is an increasing demand for future-proof, robust measurement and modeling methods. These methods are essential for evaluating the toxicity and exposure of chemicals, facilitating informed risk-based decision-making even when data on hazards and exposures are limited. It is worth noting that the need for adaptable approaches in chemical safety and sustainability efforts must also prioritize cost-efficiency and gain widespread acceptance among regulatory bodies, industry stakeholders, and the general public.

For instance, the EU has introduced the 8th Environment Action Programme, as

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<sup>1</sup>For instance, REACH, short for Registration, Evaluation, Authorisation, and Restriction of Chemicals, is an EU regulation aimed at improving chemical safety and allocating risk management responsibilities to companies operating in various sectors.

## 1.2. The Imperative for Prioritization and Toxicity Assessment

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outlined in its European Green Deal (citeregreendeal), to provide direction for European environmental policy until the year 2030. This program reinforces the EU's ambitious goal of sustainable living within planetary limits, with a forward-looking vision that extends to 2050. Central to this vision is a zero-pollution commitment, encompassing air, water, and soil quality, all while prioritizing the well-being of EU citizens. In 2021, the European Commission introduced a sustainability-focused chemicals strategy [4], which aligns with the EU's zero-pollution ambition. This strategy not only enables the evaluation of the safety and sustainability of both existing and future chemical compounds but also aims to reduce concerning substances, such as *per- and polyfluoroalkyl substances (PFAS)*, through substitution or phasing out wherever feasible. In parallel, the U.S. Environmental Protection Agency (EPA) shares a similar scientific consensus and is at the forefront of assessing the potential impacts of chemicals on human health and the environment. Leveraging advanced toxicological and exposure methods, EPA actively promotes risk reduction efforts through its own *Chemical Safety for Sustainability National Research Program*. This program builds upon the achievements of research initiatives like *ToxCast*<sup>2</sup>, *Tox21*<sup>3</sup>, and the Endocrine Disruptor Screening Program in the 21st Century (EDSP21), demonstrating a commitment to advancing chemical safety on a global scale.

### 1.2 The Imperative for Prioritization and Toxicity Assessment

Contemporary analytical techniques, including *high-resolution mass spectrometry (HRMS/MS)*, are gaining significance across various domains such as metabolomics, drug discovery, environmental science and toxicology [5]. The application of nontarget HRMS/MS has notably improved the ability to detect unidentified emerging contaminants from environmental samples, often with unknown toxicity profiles. In the past, when it comes to prioritizing unidentified compounds, the standard approach has been to use signal intensity from the fragmentation data of tandem mass spectrometry (referred to as MS/MS or MS2). We now abbreviate this as MS2 for convenience. However, this approach tends to fall short in delivering an accurate assessment of environmental exposures because MS2 signal intensity may not relate proportionally to the compound's concentration in the sample. Furthermore, and of central importance to this work, this approach overlooks the toxicological factors essential for prioritizing compounds with concerns related to environmental hazards. As a result, substances with the potential for severe ecological consequences, such as endocrine-disrupting compounds, often go undetected because of their low abundance, even though they exhibit high levels of toxicity. Hence, a pressing need exists for alternative approaches to prioritize unidentified nontarget HRMS/MS signals based on their hazard potential. Incorporating relevant toxicity considerations into the equation:

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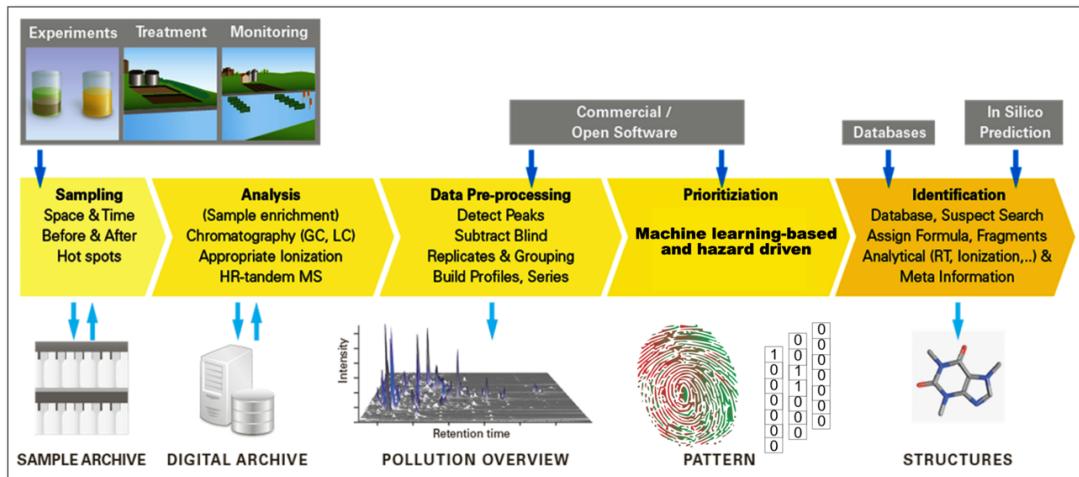
<sup>2</sup><https://www.epa.gov/comptox/toxcast>

<sup>3</sup><https://tox21.gov/>

### 1.3. Unlocking the Potential of High-Throughput Screening and Machine Learning in Toxicity Prediction

$$\text{Risk} = \text{Hazard} \times \text{Exposure} \quad (1.1)$$

augments our capacity to make well-informed decisions when evaluating the environmental risk associated with chemicals. Figure 1.1 illustrates the non-target screening with *HRMS/MS* technique and the novel prioritization approach.



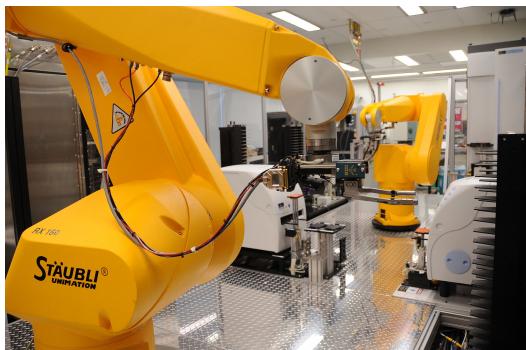
**Figure 1.1:** Schematic of the workflow used for non-target screening of environmental samples, featuring a customized prioritization step. Adapted from Figure 1 in the original source [6].

## 1.3 Unlocking the Potential of High-Throughput Screening and Machine Learning in Toxicity Prediction

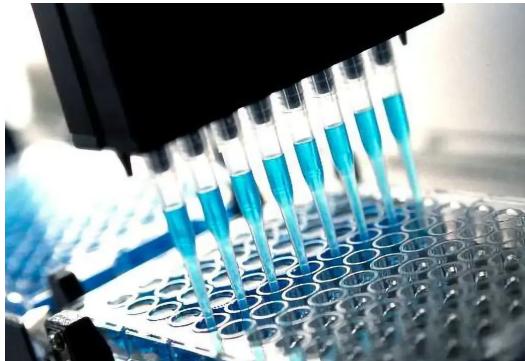
In the past few years, the use of machine learning methods has emerged as a transformative force in the field of *in vitro* toxicology, particularly in the realm of high-throughput toxicity prediction. *High-throughput screening (HTS)* has revolutionized the way toxicity is assessed by allowing thousands of *in vitro* bioassays to be conducted efficiently. This high-throughput approach, coupled with advancements in robotics and automated analysis, has generated large volumes of toxicity data, paving the way for more comprehensive assessments of chemical compounds. Alongside the rise of machine learning, this advancement has facilitated the creation of predictive models capable of forecasting compound toxicity based on their chemical structure [7]. These models can be trained on extensive datasets containing well-documented toxicity information, allowing them to learn the underlying patterns and relationships between chemical structures and target toxicity. Once trained, these models can predict the toxicity of new compounds, even if these compounds themselves have not undergone laboratory testing. This approach holds the potential to significantly

## 1.4. MLinvitroTox: A Novel Approach

reduce the time and cost associated with early-stage toxicity pre-assessment and plays a crucial role in prioritizing compounds for further in-depth testing.



**(a)** Image obtained from [8]. A robot arm retrieves assay plates from incubators and places them at compound transfer stations or hands them off to another arm that services liquid dispensers or plate readers. Efforts in the automation, miniaturization and the readout technologies have enabled the growth of HTS.



**(b)** Image obtained from [9]. Modern microtitre assay plates consist of multiples of 96 wells, which are either prepared in the lab or acquired commercially from stock plates. These wells are filled with a dilution solvent, such as *Dimethylsulfoxide (DMSO)*, along with the chemical compounds intended for analysis.

**Figure 1.2:** High-Throughput Screening (HTS)

## 1.4 MLinvitroTox: A Novel Approach

In response to the pressing need for a more hazard-driven and comprehensive assessment of environmental contaminants, Arturi *et al.* introduced *MLinvitroTox* [10], an innovative machine learning framework. This framework is integrated into a broader pipeline known as *EXPECTmine*, which also incorporates the complementary exposure aspect in the risk assessment process. The primary objective of this thesis is to collaborate with the authors to further enhance and advance this framework. MLinvitroTox leverages molecular fingerprints extracted from fragmentation spectra, marking a significant change in how the toxicity of the myriad unidentified HRMS/MS features is forecasted: While traditional *Quantitative structure-activity relationship (QSAR)* models predict bioactivities based on molecular fingerprints derived from chemical structures, MLinvitroTox was trained with supervised classification models on molecular fingerprints from chemical structures but is deployed to molecular fingerprints generated from experimentally measured MS2 spectra using *SIRIUS* and *CSI:FingerID* [11]. *SIRIUS* is a software package for annotating small molecules from nontarget HRMS/MS data, while *CSI:FingerID* is a machine-learning tool employed by *SIRIUS* to predict molecular fingerprints from fragmentation spectra. MLinvitroTox leverages streamlined machine learning techniques to predict the compounds bioactivity, respectively toxicity, ensuring a broad toxicological coverage encompassing over 400 target-specific and 70 cytotoxic endpoints, sourced from ToxCast/Tox21

data. Subsequently, the toxicity predictions generated by the framework are employed to prioritize compounds, with the flexibility to emphasize specific aspects of toxicity profiles tailored to individual preferences.

## 1.5 Objectives and Significance

The main objective of this thesis is to contribute to the development of an efficient MLinvitroTox framework for predicting compound toxicity across multiple endpoints. The goal is to enhance the integration of MLinvitroTox by creating an automated pipeline in the Python programming language. This pipeline is designed to efficiently address the inherent complexities associated with modeling and processing heterogeneous datasets. Here, the emphasis lies in enhancing the curation and filtering of toxicological data and streamlining the process, which begins with raw concentration-response series data and culminates in the generation of the final toxicity predictions. The ultimate output is expected to comprise toxicity fingerprints that encapsulate the predicted toxicity from HRMS/MS environmental samples for the relevant endpoints of interest. These generated toxicity fingerprints will offer crucial insights for the prioritization process, aiding in the identification of the most hazardous compounds present in environmental samples.

A particular limitation of the current framework is its binary approach to predicting toxicity for specific endpoints, categorizing compounds as either toxic or non-toxic without accounting for variations in toxicity severity. In the long term, it is crucial to adopt a more refined approach that can capture the nuanced continuum of toxicity. This thesis endeavors to overcome this limitation by developing a pipeline capable of forecasting toxicity across numerous endpoints, employing continuous hitcalls.

## 1.6 Thesis Structure

In the course of progressing through the subsequent chapters, insights will be provided into the materials and methods employed, focusing on the technical intricacies involved in the preparation of ToxCast/Tox21 toxicity data and their transformation into suitable inputs for the machine learning pipeline. This foundational work will establish the basis for the upcoming chapters, which will showcase the potential of MLinvitroTox. Furthermore, the framework's effectiveness is demonstrated through the validation of real-world mass spectral data from *MassBank* [12], and the examination of the implications of this research is carried out.

## 1.6. Thesis Structure

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Origin/Usage	Class	Examples	Related Issues
Industrial Chemicals	Solvents	Tetrachloro-methane	Drinking-water-quality
	Intermediates	Methyl-t-butylether	Drinking-water-quality
	Petrochemicals	BTEX (benzene, toluene, xylene)	Cancer
Industrial Products	Additives	Phthalates	Endocrine disruptors
	Lubricants	PCBs	Biomagnification
	Flame Retardants	PBDEs	
Consumer Products	Detergents	Nonylphenol ethoxylates	Endocrine effects
	Pharmaceuticals	Antibiotics	Bacterial resistance
	Hormones	Ethinyl estradiol	Feminization of fish
Biocides	Pesticides	DDT	Toxic effects and persistent metabolites
	Nonagricultural biocides	Tributyltin	Endocrine effects
Geogenic & Natural Chemicals	Heavy Metals	Lead, cadmium, mercury	Organ damage
	Inorganics	Arsenic, selenium, fluoride	Drinking-water-quality
	Taste and Odor	Geosmin	
	Human Hormones	Estradiol	Feminization of fish
Disinfection & Oxidation	Disinfection by-products	Haloacetic acids, Bromate	Drinking-water-quality
Transformation Products	Metabolites from all above	Metabolites of perfluorinated compounds Chloroacetanilide herbicide metabolites	Bioaccumulation Drinking-water-quality

**Table 1.1:** Table 2 adapted from [3]. Examples of ubiquitous water pollutants.

## Chapter 2

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# Background

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This chapter is vital for understanding the following sections of this thesis as it provides some foundational background information in toxicity testing.

### 2.1 Toxicity Testing: From In Vitro Assays and Molecular Fingerprints to Predictive Models and Beyond

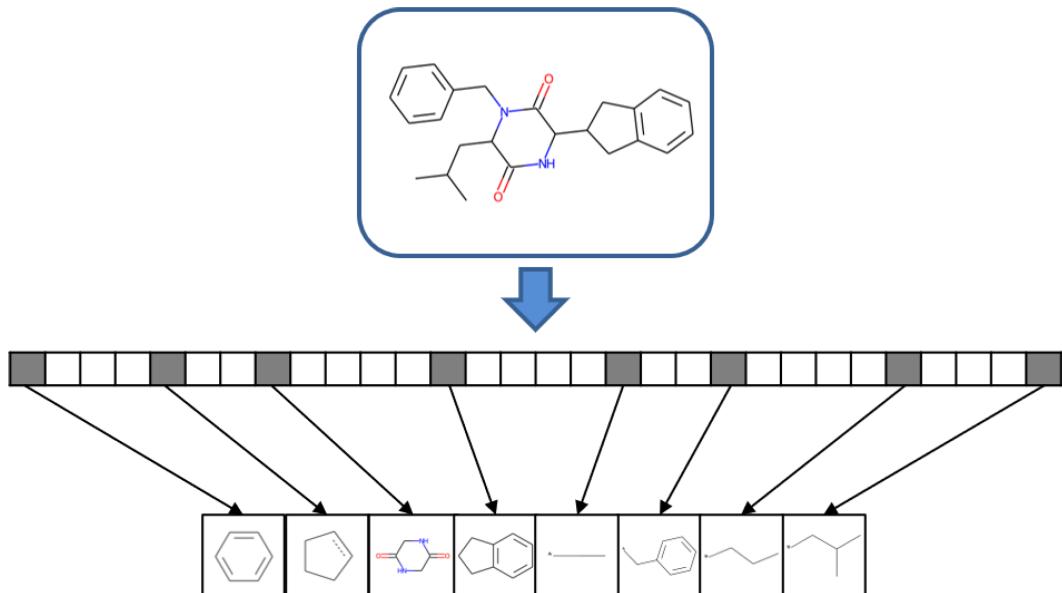
With the ever-growing amount of chemical compounds entering the environment, conducting experiments on all these compounds using traditional methods have constraints related to expense and time, but also has ethical considerations regarding animal trials.

In 2007, the *U.S. National Academy of Sciences* introduced a visionary perspective and published a landmark report, titled as *Toxicity Testing in the 21st Century: Vision and Strategy*. This report promoted a transition from conventional, resource-consuming animal-based *in vivo* tests to efficient high-throughput *in vitro* pathway assays on cells.

This transition paved the way for the realm of HTS, where a multitude of *in vitro* bioassays can be executed, complementing and improving chemical screening. This transformation is made possible by advancements in robotics, data processing, and automated analysis. As a result, this synergy has led to the generation of extensive toxicity datasets like ToxCast and Tox21.

HTS datasets, including ToxCast and other sources, have opened the door to promising applications of machine learning in predictive computational toxicology. These predictive models can be developed to screen environmental chemicals with limited availability of toxicity data, allowing for the prioritization of further testing efforts. Such models often forecast toxicity using QSARs, which are based on chemical structures encoded as descriptors like molecular fingerprints. 1D-Molecular fingerprints encode compound molecules as fixed-length binary vectors, denoting the presence (1) or absence (0) of specific substructures or functional groups.

## 2.1. Toxicity Testing: From In Vitro Assays and Molecular Fingerprints to Predictive Models and Beyond

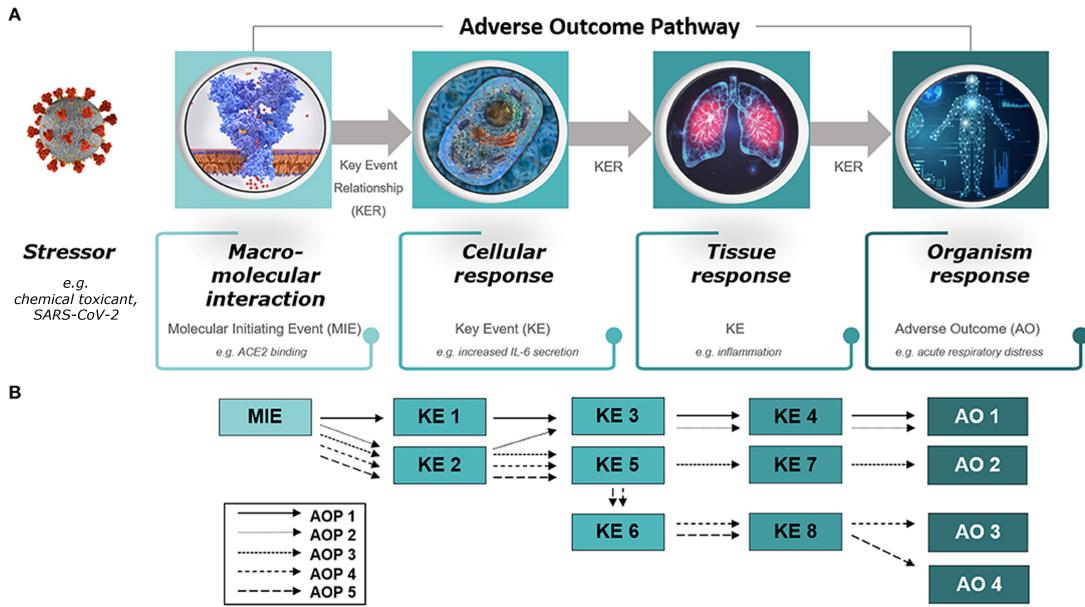


**Figure 2.1:** Figure 1 adapted from [13]. Schematic molecular fingerprint. Each bit position accounts for the presence or absence of a specific structural fragment. Bit positions are set on (set to 1, gray) if the substructure is present in a molecule, or set off (set to 0, white) if it is absent.

The utilization of molecular fingerprints for in vitro toxicity prediction is based on the assumption that molecular toxic effects result from relatively straightforward interactions between distinct chemical components and receptors during a *molecular initiating event (MEI)*. On a larger biological scale, the MEI can set a sequential chain of causally linked *key events (KE)* in motion. This occurs at different levels of biological organisation from within cells to potentially culminating in an *adverse outcome pathway (AOP)* at the organ or organism level, as depicted in Figure 2.2. The mechanistic information captured in AOPs reveal how chemicals or other stressors cause harm, offering insights into disrupted biological processes, potential intervention points but also guide regulatory decisions on next generation risk assessment and toxicity testing. The AOP framework is an analytical construct that allows an activity mapping from the presence or absence of certain molecular substructures encoded in chemical descriptors to the target mechanistic toxicity. Finally, when monitoring disruptions in toxicity pathways, *physiologically based pharmacokinetic (PBPK)* models can be leveraged to extrapolate *in vitro* findings to human blood and tissue concentrations [14].

It is crucial to emphasize that the predictions from HTS bioassays portray molecular toxicity events only at a cellular level, and their translation to adverse outcomes at higher organism levels is not necessarily guaranteed. As the scale shifts from the cellular to the organism level, the confidence in these relationships may decrease.

## 2.2. Chemical Target Toxicity vs. Cytotoxicity



**Figure 2.2:** Figure 1 adapted from [15]: Diagram of (A) an adverse outcome pathway (AOP) and (B) an AOP network. (A) An AOP starts with a molecular initiating event (MIE), followed by a series of key events (KEs) on different levels of biological organization (cellular, tissue, organ) and ends with an adverse outcome (AO) in an organism. The stressor is not part of the AOP itself.

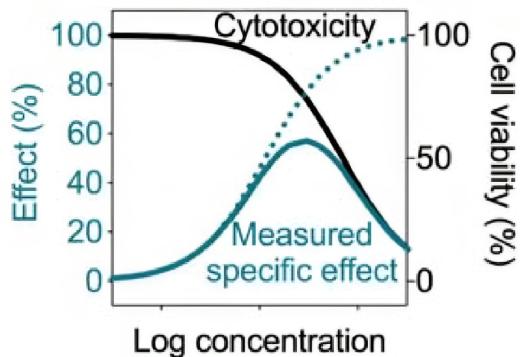
## 2.2 Chemical Target Toxicity vs. Cytotoxicity

Intuitively, an increase in chemical concentrations is expected to lead to an increase in bioactivity. However, this is not always the case. At higher doses the chemical can become cytotoxic, leading to cell deaths. In consequence, e.g., the activation of the reporter gene decreases and a signal reduction in bioactivity can be read out.

Chemical toxicity can manifest in diverse ways, falling into two major categories [16]:

- **Specific toxicity** is the result of a chemical's interaction and disruption of a specific biomolecular target or pathway, such as a receptor agonist/antagonist effect or enzyme activation/inhibition.
- **Cytotoxicity and cell stress** is the generalized disruption of the cellular machinery. Cell-disruptive processes encompass various mechanisms, such as protein, DNA, or lipid reactivity, or processes like apoptosis, oxidative stress responses or mitochondrial disruption. Cell viability can be evaluated either individually or concurrently. For instance, one approach involves evaluating it by determining the proportion of live cells within a population. This is achieved using a fluorescent dye that selectively enters living cells, as it cannot permeate the membranes of deceased cells. Consequently, the fluorescence intensity directly corresponds to cell viability.

## 2.2. Chemical Target Toxicity vs. Cytotoxicity



**Figure 2.3:** Figure 7.8 from [17]. Example of a bioassay response with cytotoxicity interference. The dotted line shows the theoretical effect but due to cytotoxicity (black line is cell viability), the measured effect has an inverted U-shape. The measured effect can additionally be confounded by the cytotoxicity burst, where even an exponential curve shape is likely for the gaining part.

It is common for compounds to exhibit target bioactivity within a limited concentration range, which coincides with a non-specific activation response in the presence of cell stress and cytotoxicity. Figure 2.3 illustrates the interference between specific toxicity and cytotoxicity.

A related phenomenon is referred to as the *cytotoxicity burst* [16] which is the suppression of many cell stress responses that are activated around a critical range of the toxicant concentration. For instance, reporter genes may be induced non-specifically near the point of cell death [17]. The ToxCast pipeline is designed to minimize *false negatives* by adopting an inclusive risk assessment approach. However, due to interference processes, the reliability of reported (specific) activities becomes uncertain, and *false positives* can occur without a thorough cytotoxicity evaluation. While some of the assay activity within this concentration range may indeed result from chemical interactions with the intended assay target, another portion does not. Nevertheless, the occurrence of false positives can be mitigated through a comparison of potency elements between the target assay endpoints and the respective viability or burst assay endpoints that quantify cytotoxic cell loss or cell stress. Previously identified false positives can be subjected to reevaluation as potential cytotoxicity interference if the probabilities indicate that the potency of the cytotoxicity assay endpoint is lower than that of the target assay endpoint.

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